# Evaluating the effect of ovarian stimulation and exogenous progesterone on CD31-positive cell density, VEGF protein, and miR-17-5p expression of endometrium immediately before implantation

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ABSTRACT

*Background:* MicroRNAs (miRNAs) form a special class of RNAs regulating endometrial functions like cell proliferation, differentiation, angiogenesis, and blastocyst implantation. In addition to providing suitable conditions for embryo development, angiogenesis is a prerequisite to natural pregnancy. The family of vascular endothelial growth factor (VEGF) and its receptors are the main physiological and pathological angiogenesis regulators in the endometrium. In the past, research has demonstrated alteration of angiogenesis and subsequent endometrial receptivity in the stimulated and luteal phase support cycles, when compared with natural cycles.

*Objective:* The objective of this study is to investigate the effect of ovarian stimulation and exogenous progesterone on the density of CD31-positive cell (Endothelial cell), VEGF protein, and miR-17-5p expression in the mouse endometrium immediately before implantation.

*Methods:* We employed ovarian stimulated and luteal phase support mice models induced by HMG/HCG and progesterone. The endometrial CD31-positive cell density was determined by immunohistochemistry (IHC) staining, the level of VEGF protein by IHC and western blot analysis, and finally the miR-17-5p expression was determined by the real-time PCR method.

*Results:* The density of endothelial cell, VEGF protein, and miR-17-5p expression increased in all of the experimental mice when compared to the control group, with the maximum increase having been seen in the group that had received progesterone after ovarian stimulation.

*Conclusion:* This research indicates that ovarian stimulation and exogenous progesterone lead to an increase in the number of endothelial cells by upregulating the VEGF protein. Moreover, except for miR-17-5p, other microRNAs and molecules are presumably involved in angiogenic pathways, thereby requiring more studies.

## 1. Introduction

MicroRNAs are small (19–25 nucleotides) non-coding RNAs that, are retained in various organisms [1]. The miRNAs genes are transcribed by the RNA polymerase II, resulting in the generation of primary miRNA (Pri-miRNA) [2]. Drosha is a nuclear RNase III enzyme removing parts of the pri-miRNA molecule to produce pre-miRNA [2]. Pre-miRNA is transported by exportin-5 into the cytoplasm to be processed by Dicer to create double-strand miRNA [3]. Duplex miRNA/miRNA* are transmitted to the RNA-induced silencing complex (RISC) to be converted into a mature, single-stranded form [4].

MicroRNAs regulate post-transcriptional gene expression [5] and affect different cellular and intracellular signaling [6]. These molecules participate in a number of biological activities including endometrial receptivity, angiogenesis, implantation, and embryo development [7].

Angiogenesis is a complex, multi-stage process through which new blood vessels are derived from pre-existing vessels [8]. Every step is controlled by numerous pro and anti-angiogenic factors [9]. VEGF, platelet-derived endothelial cell growth factor (PDGF), fibroblast growth factor$_1$ ($FGF_1$), leptin, angiopoietin, and proliferin are pro-angiogenic mediators promoting angiogenesis pathways [9]. The VEGF family is the most effective angiogenic stimulator that is proven to have a considerable impact on the endometrial vascular activity and physiological and pathological angiogenesis [10,11].

As a regenerative and dynamic tissue undergoing cyclical changes, endometrium accommodates a significant physiological angiogenesis [12]. Increased vascular permeability and angiogenesis substantially are shown to contribute to endometrial receptivity, blastocyst attachment, deciduation, and placentation [13]. Vascular development disorders are instrumental in the pathogenesis of reduced endometrial maturation and implantation failure [14].

MicroRNAs control angiogenesis by affecting the endothelial cells function and also by modulating the expression of proteins promoting or inhibiting the vessel growth [6]. miR-17-5p is a member of the miR-17-92 cluster, whose gene is on the human chromosome 13q31 [15]. Expressed in the mouse [16] and human endometrium [17], this miRNA regulates endometrial angiogenesis by targeting VEGF mRNA [18]. Under pathological endometrial conditions, an upregulation or downregulation of miR-17-5p has been detected, resulting from angiogenic disorders like endometrial cancer [19], endometriosis [20], and abortion [21].

Controlled ovarian stimulation procedures are used to achieve an appropriate number of mature follicles in the assisted reproductive technology (ART) [22]. Human chorionic gonadotropin (HCG) is a therapeutic agent used at different dosages in the ovarian stimulation protocols [23]. It has been

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reported that during the receptivity time, endometrial angiogenesis undergoes alteration in the stimulated cycles, when compared to the normal cycles [24].

Supplemental progesterone is the practice commonly used to support the luteal phase of ART cycles to help improve endometrial receptivity and implantation [25]. Progesterone can affect the density, permeability, and blood flow of endometrial vessels [26]. It has been documented that HCG [27] and progesterone modify endometrial angiogenesis by altering the VEGF protein production [28]. Moreover, these hormones adjust the endometrial microRNAs profile [29,30].

The objective of the present study is to evaluate the effect of ovarian stimulation and exogenous progesterone on the density of CD31-positive cell, VEGF protein, and miR-17-5p expression of endometrium during the preimplantation period.

## 2. Materials and methods

### 2.1. Ethics statement

The present research was approved by the Ethics Committee of the Isfahan University of Medical Sciences (Isfahan, Iran, No-397812) and all procedures involving animals were carried out in full compliance with guidelines set out on the care and use of laboratory animals.

### 2.2. Animal models and tissue preparation

Forty mature female and twenty adult male mice (NMRI, 10–12 weeks, 25–40 g) were held under standard conditions (room temperature:22 °C, 12:12 h light/dark cycle, and free access to food and water) in an animal house.

The mice were divided into the following four groups (The HMG injection day was considered as day zero in all groups): 1) control group (CO), no intervention was made. 2) ovarian stimulation group (HMG/ HCG), HMG (Shafayab Gostar Co-Iran) 7.5 IU intraperitoneal (IP), and after 48 h HCG (Pooyesh Darou Co-Iran) 7.5 IU IP was injected. 3) progesterone group (P): progesterone (Aburaihan Co-Iran) 1 mg/mouse IP received daily for 3 days 72 h after day zero, and 4) ovarian stimulation + progesterone group (HMG/HCG + P), HMG 7.5 IU IP and after 48 h, HCG 7.5 IU IP, then progesterone 1 mg/mouse IP received daily for 3 days 24 h following the HCG administration [31].

In all groups, two female mice and a male mouse were mated overnight in a cage. Mating was confirmed by the presence of sperm in a vaginal smear the following morning. It was considered the first day of pregnancy. The mice were sacrificed 96 h after the HCG injection (immediately before implantation) using anesthesia, and one-third of middle uterine horns were removed. The right horns were fixed in 10 % natural buffered formalin for IHC, and the left samples were moved into liquid nitrogen for western blot and real-time PCR analysis.

**Table 1**
Primer sequences for Real-time PCR.

| Genes | Forward | Reverse |
|---|---|---|
| U6 | CGCTTCGGCAGCACATATAC | AAATATGGAACGCTTCACGA |
| miR-17-5p | GGCAAAGTGCTTACAGTGC | GTGCAGGGTCCGAGGT |

### 2.3. Immunohistochemistry staining

Immunohistochemistry staining was used to determine the endometrial CD31-positive cell density and the level of VEGF protein. The uterine samples were fixed in 10 % natural buffered formalin, dehydrated in ethanol, embedded in the liquid paraffin, and cut into 4-μm sections. These sections were deparaffinized with xylene and rehydrated in downward concentration gradients alcohols.

Antigen retrieval was performed through boiling in the Tris-buffered saline (PH:9). The immersion of slides in 3 % hydrogen peroxide helped inhibit the endogenous peroxidase activity. Nonspecific binding was blocked in the 5% bovine serum albumin at room temperature. The sections were then incubated using the mouse monoclonal anti-VEGF primary antibody (Diagnostic Biosystems: PDM165) overnight at 4 °C, and subsequently, with HRP-conjugated goat anti-rabbit IgG secondary antibody (Protaqs:300155400) following the manufacturer's instructions. The samples were counterstained with Harris' hematoxylin. The sections were photographed using a light microscope (Olympus Corporation 40x), and the images were analyzed by the Image J software (NIH, MD, USA). CD31 was used as a marker to identify endothelial cells. This staining was carried out in line with the above-mentioned method and the mouse monoclonal anti-CD31 antibody (Zytomed system: BMS044) was applied [32].

### 2.4. Western blot analysis

The amount of the VEGF protein in the endometrium was measured by the western blot technique while the tissues were lysed using RIPA lysis buffer. Assessment of the protein concentration was performed by the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA). The proteins were separated from the SDS-PAGE gel through electrophoresis before being moved to polyvinylidene difluoride fluoride (PVDF) membrane using electrophoretic transfer. The membrane was blocked with 5% skim milk at room temperature and subsequently incubated by the rabbit anti-VEGF antibody (Biorbyte -Orb191500) overnight at 4 °C. The specific antibody complex was detected by applying the HRP- conjugated goat anti-rabbit IgG secondary antibody (Abcam- AB6721). The results were normalized to the GAPDH band intensity as internal control while, rabbit anti- GAPDH antibody (Abcam-181602) was used as the primary antibody. The bands were visualized with an ECL western blotting detection system (GE Amersham, UK). The densitometry of the protein bands was tested by the Image J software (NIH, MD, USA).

### 2.5. Real-time PCR

The miR-17-5p expression level was determined by real-time PCR. Total RNA was isolated from endometrium tissues following the Trizol Reagent instructions (Rojetecchnology Co-Iran).The RNA

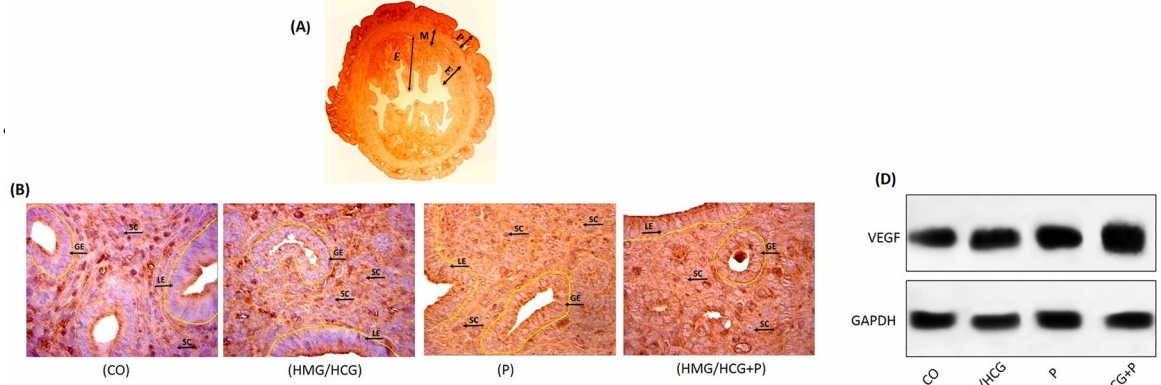


**Fig. 1.** Expression of VEGF protein in endometrium. (A) Layers of mouse uterine horn, IHC staining (4x), E (Endometrium), M (Myometrium), and P (Perimetrium). (B) IHC images displaying VEGF protein expression in endometrium (40x). (C) quantitative analysis for VEGF protein expression compared to the control group. This protein increased in all treated groups, with the maximum level detected in the HMG/HCG + P group, which was significantly different from that of the control group (* $P < 0.05$). (D) VEGF protein bands specified by western blot analysis. (E) quantitative analysis of VEGF protein in comparison with the control group. This protein was significantly upregulated in all treatment groups (*$P < 0.05$), with no significant differences among them ($P > 0.05$). All data were presented as mean ± SE and the acceptable significant difference was at $P < 0.05$. CO (control), HMG/HCG (ovarian stimulation), P (progesterone), and HMG/HCG + P (ovarian stimulation + progesterone).

purity was measured by NanoDrop 2000 (Thermo Scientific- USA). Then, cDNA was synthesized with the RT-PCR Pre-Mix Kit (Bio Fact-Korea), and the Real-Time PCR Master Mix Kit (Bio Fact- Korea) and specific miR-17-5p primers were used for the real-time PCR. Considered as the internal reference of miR-17-5p, U6 was evaluated for each sample. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The miRNA Primer Design Tool

progesterone).

software was employed to design miR-17-5p primers [33]. Table 1 presents the list of primer sequences.

### *2.6. Statistical analysis*

Data analysis was done using the SPSS (version 22) software (IBM, New York, NY, USA). The differences between the groups were evaluated using one-way ANOVA. The data were reported as the mean ± standard error. $P < 0.05$ was considered statistically significant.

## 3. Results

### *3.1. VEGF protein production increased following ovarian induction and progesterone administration*

Fig. 1A illustrates the uterine horn layers of a mouse by IHC staining.

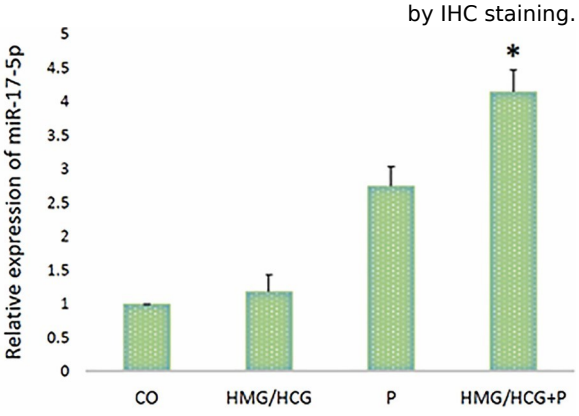


**Fig. 3.** Expression of miR-17-5p in endometrium. Based on the quantitative analysis, in all treated groups, the miR-17-5p expression was upregulated when compared to the control group, which was significant only in the HMG/HCG + P group (*$P < 0.05$). All data were presented as mean ± SE and the acceptable significant difference was at $P < 0.05$. CO (control), HMG/HCG (ovarian stimulation), P

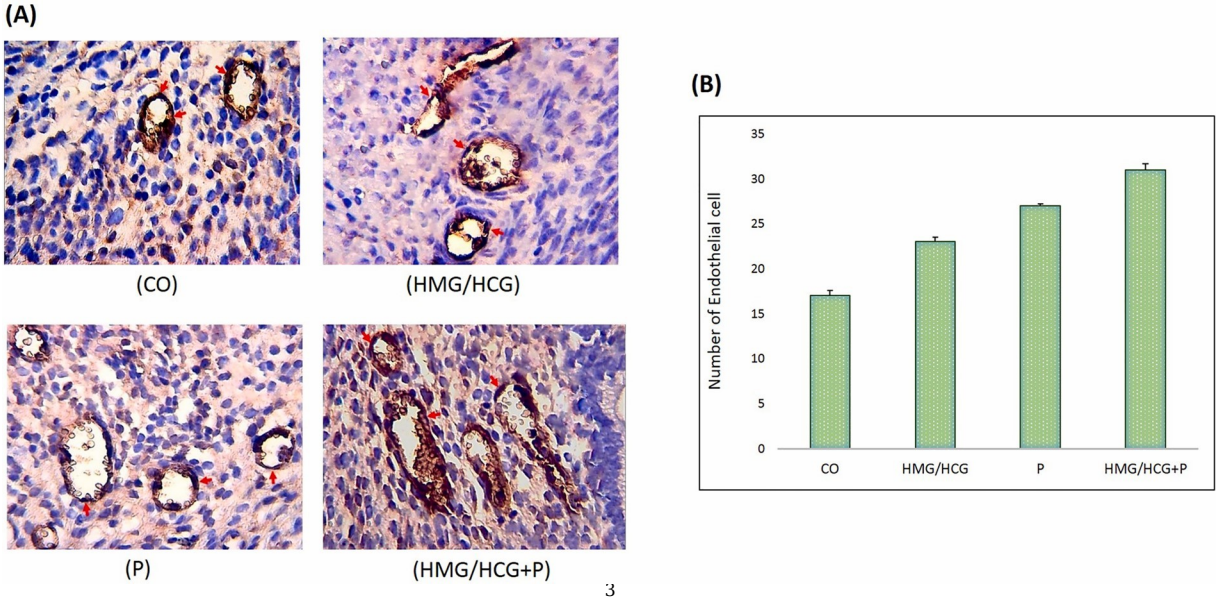


**Fig. 2.** CD31-positive cell density in endometrium. (A) Endothelial cells are shown with arrows, IHC staining (40x). (B) quantitative analysis of endothelial cells density compared to that of control group. This rate increased in all experimental groups with the highest measure found in the HMG/HCG + P group ($P > 0.05$). All data were presented as mean ± SE and the acceptable significant difference was at $P < 0.05$. CO (control), HMG/HCG (ovarian stimulation), P (progesterone), HMG/ HCG + P (ovarian stimulation + progesterone).

(progesterone), HMG/HCG + P (ovarian stimulation + progesterone).

Compared with the control group, the VEGF protein was upregulated in all treated groups (Fig. 1B). The HMG/HCG + P group showed maximum protein level, which was significantly different from that of the control group ($P < 0.05$). Besides, the other groups showed no significant difference ($P > 0.05$) (Fig. 1C).

The western blot analysis indicated that the VEGF protein concentration was significantly higher in all treated groups than in the control group ($P < 0.05$) with the major difference having been detected in the HMG/HCG + P group (Fig. 1D). No significant difference was found between the treated groups ($P > 0.05$) (Fig. 1E). Overall, the IHC and western blot revealed that HMG/HCG and progesterone elevated the expression of VEGF protein, thereby inducing endometrial endothelial cell proliferation and angiogenesis.

### *3.2. VEGF protein expression improves endothelial cells density in endometrium*

CD31 was used as a marker to identify endothelial cells in the tissue sections. IHC findings showed, the number of CD31-positive cells was higher in all experimental groups than in the control group (Fig. 2A). Nonetheless, there was no noticeable difference between the experimental groups ($P > 0.05$) (Fig. 2B). In the progesterone treated mice, the vascular density was improved compared to that of the control and HMG/HCG groups. The highest endothelial cell density belonged to the HMG/HCG + P group, indicating that the administration of progesterone after ovarian induction stimulates the endometrial angiogenesis.

### *3.3. miR-17-5p expression upregulated after ovarian induction and progesterone administration*

The miR-17-5p expression was determined by the real-time PCR method. The results revealed that compared to the control group, miR- 17-5p was upregulated in all treated groups, which was significant only in the HMG/HCG + P group ($P < 0.05$). No significant difference was observed between the experimental groups ($P > 0.05$) (Fig. 3).

## 4. Discussion

Endometrial receptivity is a prerequisite for blastocyst attachment and successful implantation [34]. Ovarian stimulation affects the endometrial angiogenesis index [35] growth factors (VEGF and TGF-β1) [36], the concentration of interleukins (IL-12, IL-17, and IL-5) [37] proteins (Integrins, Glycodelin-A, and L-selectin ligand) [38] genes expression (Dkk-1, EGF, and Eotaxin) [37], normal hormones profile (progesterone and estrogen) [39], and the profile of microRNAs [40], bringing about changes in the endometrial receptivity [41].

This study demonstrated that VEGF protein expression and the number of CD31- positive cells were higher in the HMG/HCG group than in the control group. During the preimplantation period, VEGF plays a significant role in enhancing angiogenesis and vascular permeability [42].VEGF mRNA and protein are found in the endometrial luminal epithelium, stromal cells, and glands [43]. Furthermore, HCG receptors have been identified in the endometrial epithelium and stromal cells, binding HCG with its receptors triggers these cells to hyperproliferative, and elevated VEGF in the endometrium [44].

The expression of angiogenic factors (VEGF, FGF2, and CCL2) is reported to have altered following HCG administration to human endometrial epithelial and stromal cells, indicating HCG direct impact on the VEGF protein expression [45]. WS Rahmatullah et al. demonstrated that ovarian stimulation increased the VEGF protein level, the endometrial thickness, and the vascular blood flow [46]. miRNAs are involved in the regulation of endothelial cells proliferation, differentiation, and migration during angiogenesis [47]. miR-17-5p targets VEGF mRNA and negatively regulates the VEGF protein expression by directly binding to the 3′ UTR of VEGF mRNA [48].

In the present study, compared with the control group, the HMG/HCG group saw miR-17-5p upregulation. In case this miRNA bound to 3′ UTR of VEGF mRNA, the VEGF protein expression would be expected to be reduced [49]. That is while, western blot and IHC indicated a VEGF protein increase.

It has been documented that miRNAs interact with the 5′ UTR and coding sequence (CDS) region of target mRNAs that have powerful functional effects and play a transcriptional regulating role [50]. Recent studies showed that miR-17-5p binds to the 3′UTR, 5′UTR, and the CDS region of its target mRNAs [51,52]. As noted in foregoing studies and probably in the present study, miR-17-5p binds to the 5′UTR or CDS region of VEGF mRNA and increases the VEGF protein expression.

Furthermore, each mRNA is known to be regulated by multiple miRNAs [53]. It has been observed that compared with natural cycles, some miRNAs such as miR-424, miR-503, miR-34-5p, and miR-34b were dysregulated in the stimulated cycles [29]. In another study, the has-miR-520 g, has-miR-369-3p, and has-miR-186 expression changed following ovarian stimulation [40]. In these studies, miR-34-5p, miR-423-5p, miR-34b, miR-503 [29] and miR-520 g, miR-369-3p, and miR-186 control the VEGF mRNA expression [40].

Based on the findings of foregoing studies, in the present study, other miRNAs could be due to the upregulation of VEGF in the mice endometrium, which would require further investigation. microRNAs target VEGF mRNA in the mouse using miRWalk2.0 databases including: mmu- miR-150-5p, mmu-miR-340-5p, mmu-miR-466f-3p, mmu-miR-329-3p, mmu-miR-466i-3p, mmu-miR-362-3p, mmu-miR-20b-5p, mmu-miR-93- 5p, mmu-miR-20a-5p, mmu-miR-15a-5p, mmu-miR-106a-5p, mmu- miR-539-3p, mmu-miR-381-3p, mmu-miR-499-5p, and mmu-miR-16-5p (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/).

Exogenous progesterone is a steroid hormone commonly used to support luteal phase and reduce failure of implantation [54]. Progesterone adjusts the endometrial receptivity through controlling

interleukins secretion (IL-15 and IL-8), angiogenic factors (VEGF, SDF1,

**Table 2**
Several targets of miR-17-5p associated with VEGF protein.

| Target | Reference |
|---|---|
| $Fgf_{10}$ (Fibroblast growth factor 10) | [66] |
| $Bcl2l_{11}$(Bcl-2-like protein 11) | [67] |
| $Bmp_4$ (Bone morphogenetic protein 4) | [68] |
| $Map_4$ (Microtubule-associated protein 4) | [69] |
| $Celf_2$ (CUGBP Elav-like family member 2) | [70] |

investigation, miR-17-5p was upregulated in the progesterone group when compared to the control and HMG/HCG groups. This upregulation may be attributed to what was used to explain the role of the HMG/HCG group in of VEGF protein upregulation.

The maximum level of miR-17-5p, VEGF protein expression, and endothelial cell density belonged to the HMG/HCG + P group. Previous studies showed that in the stimulated cycles, ovarian induction resulted in the supraphysiological level of serum $E_2$ [63] and $P_4$ during the late follicular phase [64]. The elevated steroid concentration may entail alterations

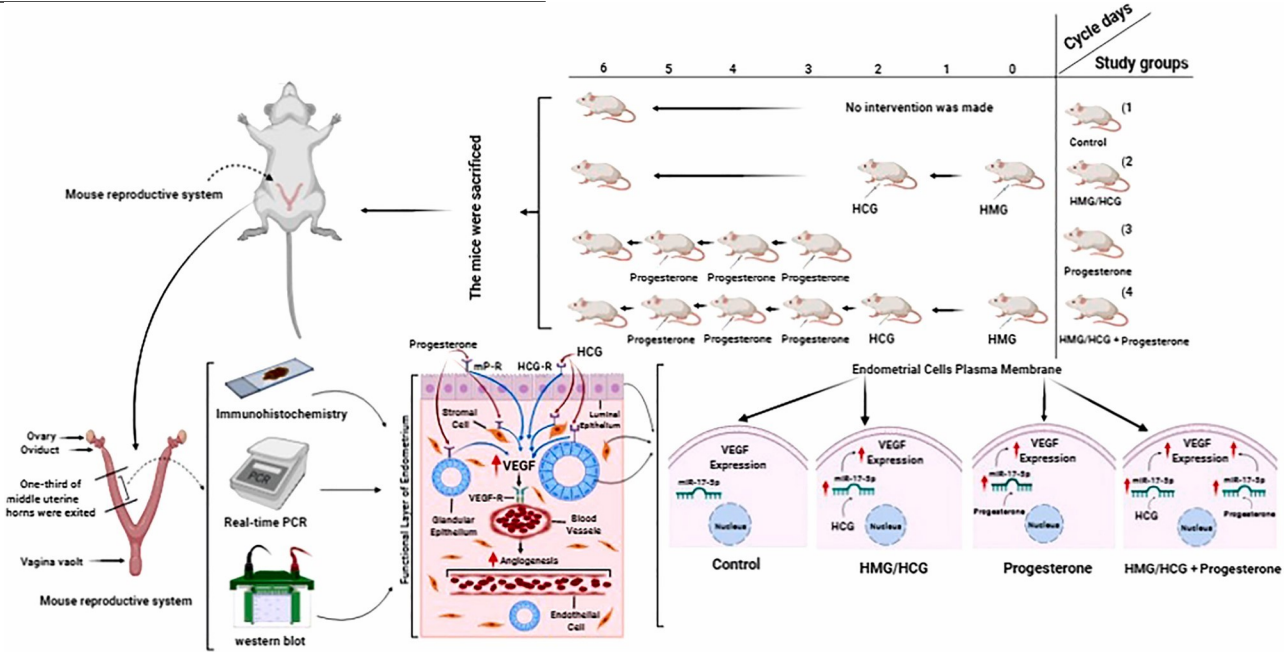


**Fig. 4.** Schematic diagram of miR-17-5p upregulation and increased endometrial angiogenesis in stimulated and luteal phase support cycles. mPR (membrane progesterone receptor), HCG-R (Human chorionic gonadotropin–recepotor), VEGF-R (Vascular endothelial growth factor-receptor).

and angiogenin) [55], and genes expression (IGFBP1, C/EBPβ, PLZF, MIG-6, and CRISPLD2) [56].

The findings of this investigation showed that CD31-positive cell density and VEGF protein expression were higher in the progesterone group than in the control and HMG/HCG groups.

Since progesterone receptors are found in the endothelial cells, they affect the endothelial junctional proteins (CDH5, PECAM-1, and CLDN5), endothelial cell proliferation, migration, and vessels permeability [57].

Besides, the progesterone binding with its nuclear receptors in the endometrial cells stimulates the production of VEGF and proliferation of endothelial cells [58,59].

Exogenous progesterone considerably affects the endometrial miRNA profiles [40]. After upregulation or downregulation of miRNAs following progesterone administration, the endometrial receptivity changes through associated target genes [40].

It has already been shown that several mature miRNAs (mmu-miR- 192-5p, mmu-miR-320-3p, mmu-let-7a-5p, mmu-miR-21a-5p, and mmu-miR-29a-3p) and their targets (VEGF, Tgfb2, Tfdp2, Slc2a3, Slc2a1, Pdgfra, Mmp15, Mcl1, and Lamc1) were upregulated in the mouse endometrial epithelial cells through progesterone [60]. Progesterone upregulates miR-133a [61] and downregulates miR-200 expression in the endometrium [62]. In this

in the morphological, molecular, chemical, and gene expression associated with the receptivity of uterine [64], including the expression of VEGF protein [65] and the profile of miRNAs [63].

Given the findings of the present research and the foregoing studies, we suggest that the reported results may stem either from increased steroid hormones following ovarian stimulation, or the synergistic effects of HCG and progesterone.

Furthermore, it has been proven that each miRNA has several target mRNAs [53]. For instance, miR-17-5p has multiple targets (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) associated with the VEGF protein (Table 2). Therefore, this miRNA can affect other targets and can indirectly change the expression of VEGF protein and angiogenesis, That necessitates conducting complementary studies. (Fig. 4 presents the graphical illustration of the results and analysis of this study).

## 5. Conclusion

The results of the present research conclude that ovarian stimulation and progesterone administration enhance expression of the VEGF protein and angiogenesis, which is key to endometrial receptivity. Moreover, these hormones adjust the miRNAs profile such as miR-17-5p, which may affect

different biological pathways, except for angiogenic ones, and alter the endometrial receptivity following embryo implantation.

## Authors contributions

B. R. designed and supervised the study, M.Sh. led the molecular parts and analyzed the data, and S.S. conducted the experiments. All the authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors report no declarations of interest.

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